Enhancement of transglutaminase activity by NMR identification of its flexible residues affecting the active site

Nobuhisa Shimba, Mina Shinohara, Kei-ichi Yokoyama, Tatsuki Kashiwagi, Kohki Ishikawa, Daisuke Ejima, Ei-ichiro Suzuki*

Central Research Laboratories, Ajinomoto Co., Inc., 1-1 Suzuki-cho, Kawasaki-ku, Kawasaki-shi 210-8681, Japan

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Abstract Incorporation of inter- or intramolecular covalent cross-links into food proteins with microbial transglutaminase (MTG) improves the physical and textural properties of many food proteins, such as tofu, boiled fish paste, and sausage. By using nuclear magnetic resonance, we have shown that the residues exhibiting relatively high flexibility in MTG are localized in the N-terminal region; however, the N-terminal region influences the microenvironment of the active site. These results suggest that the N-terminal region is not of primary importance for the global fold, but influences the substrate binding. Therefore, in order to increase the transglutaminase activity, the N-terminal residues were chosen as candidates for site-directed replacement and deletion. We obtained several mutants with higher activity, del1–2, del1–3, and S2R. We propose a strategy for enzyme engineering targeted toward flexible regions involved in the enzymatic activity. In addition, we also briefly describe how the number of glutamine residues in a substrate protein can be increased by mixing more than two kinds of TGases with different substrate specificities. © **2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

Key words: Transglutaminase; Nuclear magnetic resonance; Flexible region; Site-directed mutagenesis; Protein engineering

1. Introduction

Transglutaminases (TGase: protein-glutaminase γ-glutamyltransferase, EC 2.3.2.13) are a family of enzymes that catalyze the displacement of the amide ammonia at the γ-position in glutamine residues by replacing it with another amine, usually an ε-amino group from a suitable lysine residue [1–4]. The formation of ε-(γ-glutamyl)lysine isopeptide bonds results in both intra- and intermolecular cross-linking of proteins, leading to polymerization. The incorporation of inter- or intramolecular covalent cross-links into food proteins with microbial transglutaminase (MTG) improves the physical and textural properties of many food proteins, such as tofu, boiled fish paste, and sausage [5–7].

It has been reported that the polymerization of several proteins with MTG progressed rapidly in comparison with the reaction rate with guinea pig liver transglutaminase (GTG) [8]. Moreover, we have already shown that GTG and red sea bream liver transglutaminase (FTG) would be restricted with regard to this industrial use, due to their high substrate specificities [9]. On the other hand, the reaction rate for Ser-MTG, which is an MTG variant lacking the N-terminal aspartic acid residue, was higher than that observed for MTG, even though their substrate specificities are identical to each other [9]. At the present time, MTG and its variant are the preferred enzymes for industrial use. Therefore, MTG variants with higher activity are expected not only to be useful in the present field, but also to extend the scope of other industrial applications to biological materials, drugs, and so on. Because the amount of the enzyme in order to change the food texture can be decreased, leading to cost cutting, and it is possible to apply other proteins not to be reacted by the present enzyme.

The proposed TGase reaction mechanism is based on the crystal structure of factor XIII [10,11]. At first, the γ-carboxyamide groups in glutamine residues interact with TGases and, subsequently, the primary amino groups of a variety of amines or the ε-amino groups of lysine residues are involved in the formation of new covalent bonds. The reactivity of a glutamine residue depends on the type of TGase and the microenvironment formed by its surrounding residues in the substrate protein. On the basis of the proposed reaction mechanism, we have already developed an enzymatic labeling technique (ELT), in which ^{15}N nuclei are incorporated into the γ-carboxyamide groups of the glutamine residues in arbitrary proteins [12]. In addition, we have reported a novel method using ELT and nuclear magnetic resonance (NMR) detection techniques to determine the substrate specificities for glutamine residues and the reaction rates of TGases simultaneously [9]. The method is quite useful for comparing the existing TGases and for screening new TGases or TGase variants, and will hereafter be referred to as the NMR-based screening method. On the other hand, the calorimetric hydroxamate procedure is another method for measuring TGase activity, which uses a small peptide including a glutamine residue, *N*-carbobenzoxy-L-glutaminyl-glycine [13]. This method is convenient to estimate the reaction rate for the small peptide, but is not adequate for an analysis of the substrate specificity.

We are also studying the structure of MTG by NMR spectroscopy, in an effort to understand the process of substrate recognition. We are focusing on the experimental results that

*Corresponding author. Fax: (81)-44-210 5872.
E-mail address: eiichiro_suzuki@ajinomoto.com (E.-i. Suzuki).

Abbreviations: TGase, transglutaminase; MTG, microbial transglutaminase; ELT, enzymatic labeling technique; GTG, guinea pig liver transglutaminase; FTG, red sea bream liver transglutaminase

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the thiol group of Cys64 in MTG is essential for the enzymatic activity, and that the reaction rate for Ser-MTG is higher than that for MTG [9,14]. To obtain as much relevant information concerning the MTG structure and function as possible, we have chosen strategies that rely on the use of existing mutants and stable isotope labeling. High-level expression systems for MTG variants with an additional methionine residue and with an aspartic acid residue deletion in the N-terminus were reported previously [15]. Using these systems, we embarked on research to understand the structural role of the N-terminal residues, and to obtain more efficient enzymes by site-directed mutagenesis.

In the present work, dynamic filtering by the use of a CPMG pulse train [16] was applied for the extraction of resonances originating from flexible parts of the MTG molecule. In addition, the effects of the flexible region on the active site, Cys64, in MTG were revealed by heteronuclear 2D NMR experiments of MTG selectively labeled with ^{15}N cysteine. On the basis of these NMR structural studies, we obtained several mutants, including some with higher activity than the wild-type MTG. Finally, we briefly discuss a strategy for enzyme engineering based on the NMR study.

2. Materials and methods

2.1. Sample preparation for the NMR study

The MTG variant with an additional N-terminal methionine residue, which is encoded by a start codon, is referred to as Met-MTG. The MTG variant lacking the N-terminal aspartic acid residue has a serine residue at the N-terminus, and thus hereafter is referred to as Ser-MTG. Recombinant Met-MTG and Ser-MTG were expressed and purified as described previously [15].

L-^{15}N-Cysteine was purchased from CIL. To incorporate the ^{15}N-labeled cysteine into Met-MTG and Ser-MTG, 50 mg of L-^{15}N-cysteine/l was added to the M9 medium. Expression and purification of the MTG variants labeled with ^{15}N-cysteine were performed as described previously [15].

2.2. Site-directed mutagenesis

All basic recombinant DNA procedures, such as isolation and purification of DNA, restriction enzyme digestion, and transformation of *Escherichia coli*, were performed as described by Sambrook et al. [17]. Mutagenesis was carried out on the Met-MTG gene (pUCTRPMTG-02) [15]. Site-directed mutagenesis was accomplished using a Quick change mutagenesis kit (Stratagene) according to its protocol. Mutations were confirmed by sequencing by the dideoxynucleotide chain termination method with a Dye Terminator Cycle sequencing kit (Perkin-Elmer) and a DNA sequencer (model 373A, Perkin-Elmer). Synthesized universal and fragment-specific oligonucleotides for each clone were used as primers.

E. coli BL21 DE3 was used as the host strain, and the production medium (15.1 g of $Na_2HPO_4{\cdot}12H_2O$, 3 g of KH_2PO_4, 8 g of casamino acids, 5 g of NH_4Cl, 0.2 g of yeast extract, 2 mg of thiamin·HCl, 0.5 g of NaCl, 0.5 g of $MgSO_4{\cdot}7H_2O$, 14.5 mg of $CaCl_2{\cdot}2H_2O$, and 5 g of glucose/l) containing ampicillin (50 μg/ml) was used for the culture of *E. coli* transformants. *E. coli* BL21 DE3 transformants harboring each plasmid were cultured and harvested as described previously [15]. The proteins were purified by ion-exchange column chromatography as described previously [15].

2.3. NMR measurements

NMR experiments were performed on a Bruker DMX600 spectrometer equipped with a triple resonance probe head with *XYZ* triple-axis gradient coils. All spectra for the structural study were recorded at 308 K. The ^{1}H and ^{15}N chemical shifts were calculated from the resonance of the solvent H_2O as 4.68 ppm [18]. For data processing and analysis, XWINNMR (Bruker), nmrPipe [19], and PIPP [20] were used.

Protein solutions were concentrated by ultrafiltration to a final volume of 400 μl in 20 mM sodium phosphate buffer, pH 6.0, in 95% H_2O/5% D_2O. A 5 mm NMR sample tube was used with a final protein concentration of 0.2–0.3 mM. For the selective detection of resonances with longer spin–spin relaxation times, a conventional CPMG pulse train $\pi/2$–$(\tau$–π–$\tau)_n$ with a delay time of 10.5, 21 or 31.5 ms was used [16]. The CPMG spectra were recorded with 8192 data points and spectral widths of 8400 Hz. All FIDs were multiplied

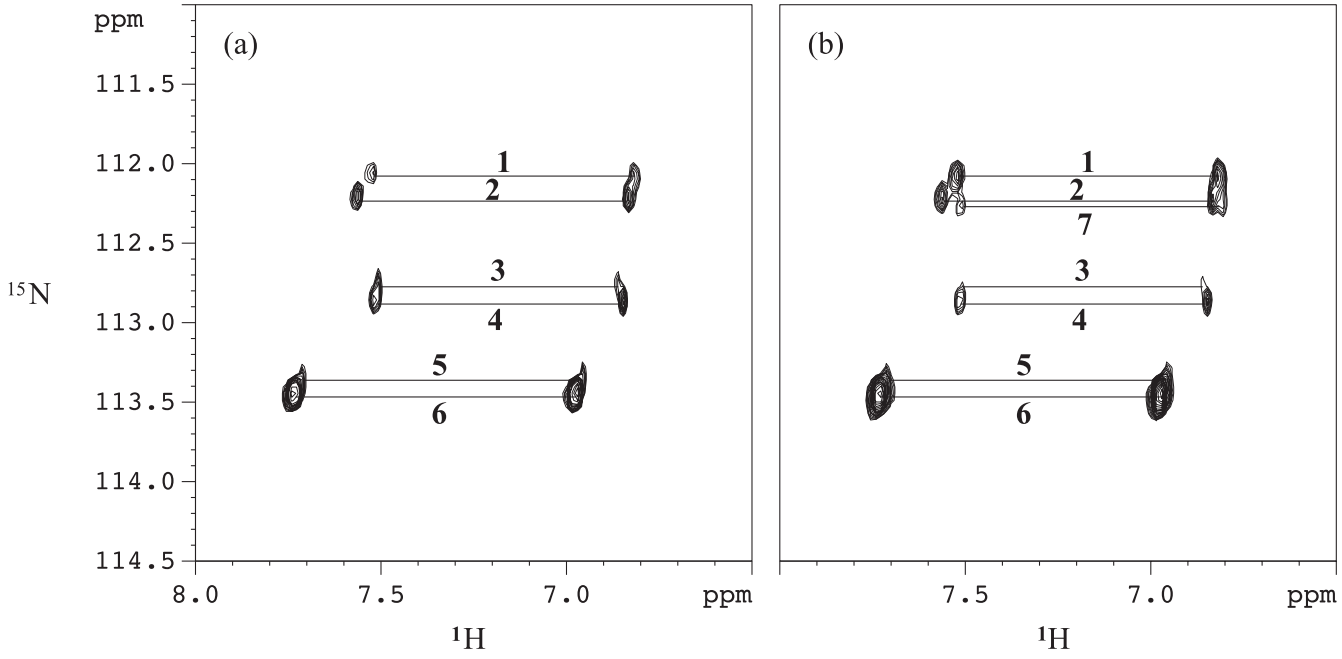

Fig. 1. a: The ^{1}H–^{15}N HSQC spectrum of ovalbumin labeled with ^{15}N in the presence of the wild-type MTG. Observed cross-peaks labeled with the wild-type MTG are numbered 1–6. The sums of the intensities for the pair of signals indicated by 6 and those for the corresponding signals in ovalbumin reacted with del1–2, del1–3, S2R and Ser-MTG are plotted against the reaction time. Signal intensities were fitted using a single exponential model function for the estimation of the TGase activities shown in Table 1. b: The ^{1}H–^{15}N HSQC spectrum of ovalbumin labeled with ^{15}N in the presence of the wild-type MTG and FTG. The glutamine residue numbered 7 was newly labeled by adding FTG.

by an exponential window function prior to Fourier transformation. The HSQC spectra were recorded with spectral widths of 8400 Hz for ^{1}H and 1400 Hz for ^{15}N. The pulse sequence for the HSQC spectra was as described by Bodenhausen and Ruben [21]. The WATERGATE water suppression scheme with the 3–9–19 refocusing pulse was incorporated into the reverse INEPT step [22]. A total of 2048 data points were used in the t_2 dimension, and 100–200 transients were acquired for the t_1 points. Prior to 2D Fourier transformation, the acquired data were multiplied by Gaussian functions in t_2 and t_1, and were zero-filled to yield a 1024 (F_2)×512 (F_1) matrix of the real data points.

2.4. Calorimetric hydroxamate procedure

The TGase activities were measured by the calorimetric hydroxamate procedure using *N*-carbobenzoxy-L-glutaminyl-glycine as described previously [13]. One unit was defined as the formation of 1 μmol of hydroxamic acid/min.

2.5. NMR-based screening method

Ovalbumin (chicken egg albumin), which is a glycoprotein (43 kDa) including 14 glutamine residues, was used as the substrate protein [9]. In order to compare the substrate specificities and to estimate reaction rates, solutions of 2.3 mM ovalbumin were prepared by adding 5 mM $CaCl_2$, 200 mM $^{15}NH_4Cl$, and 1 μM wild-type MTG, its variants, or other TGases in 20 mM sodium phosphate buffer, pH 6.0, in 95% H_2O/5% D_2O. After mixing gently, 500 μM of the protein solution was immediately placed into a 5 mm NMR tube for the NMR measurements. The probe temperature was 310 K throughout the experiments.

The ^{1}H–^{15}N HSQC spectra in the presence of MTG variants were measured continuously 14 times, to compare their substrate specificities and to estimate their reaction rates. Continuous measurements were started within 20 min after the addition of the enzyme, and each measuring time was about 135 min. A representative ^{1}H–^{15}N HSQC spectrum is shown in Fig. 1a, and the series of peak intensities, labeled 6, were extracted in a set of 2D data with the utility in nmrPipe, and were plotted as described previously [9]. In the present paper, one unit was defined as the labeling of 1 μmol of the glutamine residue labeled 6/min.

3. Results and discussion

Assignments of the ^{1}H resonances originating from the N-terminal region were made on the basis of spectral comparisons between Met-MTG and Ser-MTG. The four resonances at 2.08, 2.12, 2.59, and 2.70 ppm, observed in the difference spectrum, were assigned to Met(−1)-Hε, Met(−1)-Hβ, Met(−1)-Hγ, and Asp1-Hβ, respectively, in the N-terminal region that is deleted in Ser-MTG (Fig. 2a–c). Resonances originating from the α protons in Met(−1) and Asp1 could not be observed in the difference spectrum, because of the severe overlap with the H_2O resonance.

For the selective detection of resonances originating from flexible parts of the Met-MTG molecule, the CPMG pulse train with a delay time of 10.5, 21, or 31.5 ms was employed. The line-widths of the ^{1}H resonances for Met-MTG are usually of the order of 50 Hz at the observation frequency of 600 MHz. Under the present CPMG condition with the delay time of 10.5, 21, or 31.5 ms, most of the resonances originating from the non-flexible portions are suppressed by the use of the CPMG pulse train. In the CPMG spectrum of Met-MTG, several resonances, including those from Met(−1)-Hε, Met(−1)-Hβ, Met(−1)-Hγ and Asp1-Hβ, survived (Fig. 2d–f). These results indicate that the residues exhibiting relatively high levels of flexibility in the Met-MTG molecule are localized in the N-terminal region.

Next, the effect of the deletion of the N-terminal region was examined with regard to the enzymatic activity. Fig. 3 shows the ^{1}H–^{15}N HSQC spectra of Met-MTG and Ser-MTG labeled with ^{15}N-cysteine. We have assigned these resonances to Cys64 in Met-MTG and Ser-MTG, respectively, since only one cysteine residue, Cys64, is included in each molecule. The deletion of the Met(−1) and Asp1 residues in the N-terminal region induced a slight chemical shift change of the peak from Cys64, in which the thiol group is essential for enzymatic activity. These results show that the N-terminal region influences the static or dynamic structure of the active site in MTG.

Taking the flexibility of the N-terminal region into consideration, we noticed that the N-terminal region is not of primary importance for the global fold. In other words, the global fold does not seem to be destroyed by the replacement and deletion of several N-terminal residues. On the other hand, the N-terminal region has a slight effect on the structure of the active site, suggesting that the N-terminal region is involved in the approach and/or binding of the substrate.

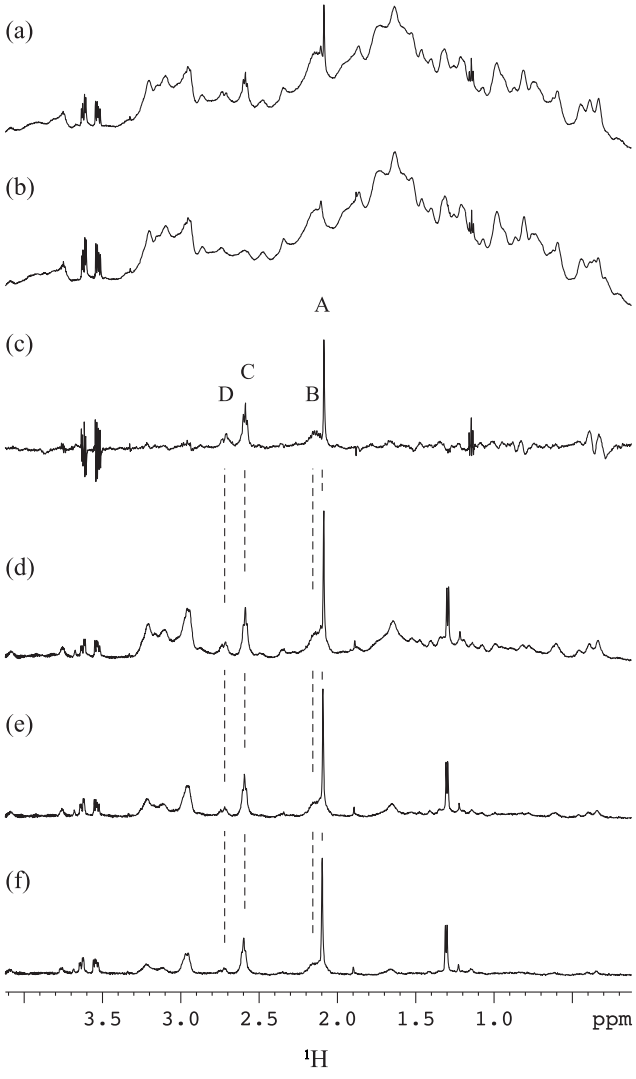

Fig. 2. ^{1}H NMR spectra of (a) Met-MTG and (b) Ser-MTG, and (c) their difference spectrum. Peaks in the difference spectrum are assigned to A: Met(−1)-Hε, B: Met(−1)-Hβ, C: Met(−1)-Hγ, and D: Asp1-Hβ. The ^{1}H spectra of Met-MTG were obtained by using a CPMG pulse sequence with a delay time of (d) 10.5 ms, (e) 21 ms, or (f) 31.5 ms. It should be noted that one of the signals, observed at 1.3 ppm in the spectra (d), (e), and (f), originated from contamination.

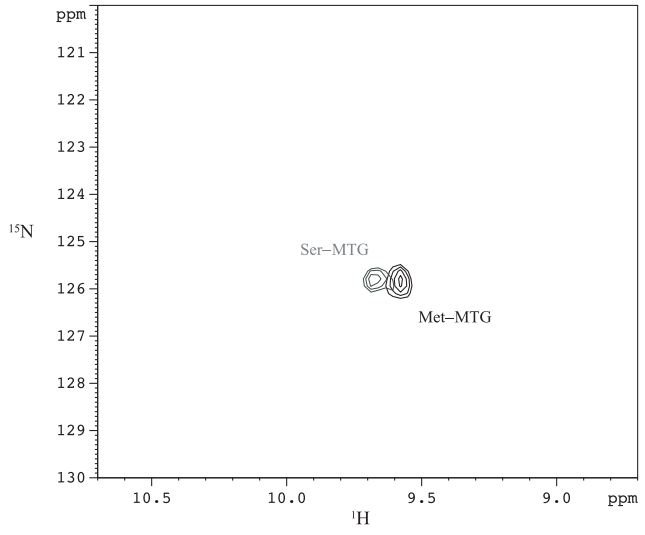

Fig. 3. ^{1}H–^{15}N HSQC spectra of Met-MTG and Ser-MTG labeled with ^{15}N-cysteine are shown in black and gray, respectively. Resonances in both spectra are assigned to Cys64 in Met-MTG and Ser-MTG, respectively.

As a practical matter, the reaction rates of the wild-type MTG and Ser-MTG have already been compared, using ovalbumin as the substrate protein [9,15]. The reaction rate for Ser-MTG is higher than that observed for MTG, at least for some glutamine residues in ovalbumin, even though their substrate specificities are identical. Therefore, in order to increase the TGase activity, the N-terminal residues were chosen as candidates for replacement and deletion, which would disturb the substrate binding, and would retain the global fold. To minimize the volume of the N-terminal region, the following five mutants were designed: del1–2, del1–3, del1–4, and del1–5 (Table 1). In addition, we designed three mutants: Ser2→Arg (S2R), Ser2→Asp (S2D), and Ser2→Tyr (S2Y), to change the charged state of the N-terminal region (Table 1).

We compared their substrate specificities for the glutamine residues in ovalbumin, and estimated the reaction rates by using the signals corresponding to one of the glutamine residues. Six pairs of cross-peaks, like those in Fig. 1a, were observed in all of the ^{1}H–^{15}N HSQC spectra in the presence of del1–2, del1–3, S2R, S2D, and S2Y, showing that their substrate specificities are identical to each other (data not shown). However, the reaction rates for del1–2, del1–3, and S2R are higher than that observed for the wild-type MTG and, especially, the reaction rate for del1–3 exceeds that for Ser-MTG (Table 1). The activities based on the calorimetric hydroxamate procedure were also measured, as shown in Table 1. This result is basically consistent with the reaction rates estimated using the NMR-based screening method.

The increased activities of del1–2 and del1–3 suggest that the flexible N-terminal two residues are not involved in the substrate binding. On the other hand, the other deletion mutants, such as del1–4 and del1–5, lost the TGase activity, which suggests that the MTG variants lacking more than three N-terminal residues were not correctly folded. The third residue and its subsequent residues are important for the global fold and/or the TGase activity, although the flexible region exists in the N-terminus. The S2R mutant, which was designed to reduce the number of negative charges in the N-terminal region, also displayed higher activity. The activities of Ser-MTG and del1–2, which have reduced negative charges by the deletion of Asp1, were also increased. These results suggest that an excess of negative charges in the N-terminal region is not preferable for substrate binding.

Inconsistent values for the activities of S2D and S2Y were obtained, as estimated with the NMR-based screening method and the calorimetric hydroxamate procedure. In the case of the calorimetric hydroxamate procedure, the replacement of Ser2 might have a small effect, since a small peptide, CBZ-Gln-Gly, was used as the substrate. On the other hand, a relatively larger substrate protein, such as ovalbumin, was used in the NMR-based screening method. On the basis of this method, the replacement of a broad region in MTG affects the activity on account of the wider interface between TGase and its substrate protein. This would be the reason for the discrepancy between both methods.

We have already elucidated the difference in the substrate specificities among MTG, GTG, and FTG, and classified them based on the resonances of the ^{15}NH signals, using the NMR-based screening method [9]. In addition to the reaction rate increase, we also noticed that the number of glutamine residues in the substrate protein could be increased by mixing more than two kinds of TGases. Six pairs of cross-peaks were observed in the presence of MTG, showing that the corresponding γ-carboxyamide groups of the glutamine residues in ovalbumin reacted with MTG (Fig. 1a). On the other hand, seven pairs of cross-peaks were detected in the presence of both MTG and FTG (Fig. 1b). The glutamine residue 7 was newly labeled by adding FTG, revealing that when ovalbumin is the substrate protein, many glutamine residues may

Table 1
Amino acid sequences of the N-terminal regions in MTG and its variants, and their TGase activities determined by the NMR-based screening method and the calorimetric hydroxamate procedure

	Amino acid residues in the N-terminus								NMR-based screening method ($\times 10^{-2}$ U/mg)[a]	Calorimetric hydroxamate procedure (U/mg)[a]
Residue number	−1	1	2	3	4	5	6	7		
Wild-type MTG		D	S	D	D	R	V	T	5.5 ± 0.2	26.3 ± 0.2
Met-MTG	M	D	S	D	D	R	V	T	5.6 ± 1.0	26.3 ± 0.1
Ser-MTG			S	D	D	R	V	T	8.9 ± 0.9	28.9 ± 0.7
del1–2			M	D	D	R	V	T	10.3 ± 0.5	30.7 ± 1.5
del1–3				M	D	R	V	T	14.7 ± 1.3	34.1 ± 1.0
del1–4					M	R	V	T	< 2.0	< 1.0
del1–5						M	V	T	< 2.0	< 1.0
S2R	M	D	R	D	D	R	V	T	6.4 ± 0.6	32.5 ± 0.4
S2D	M	D	D	D	D	R	V	T	< 2.0	28.0 ± 0.5
S2Y	M	D	Y	D	D	R	V	T	< 2.0	30.5 ± 1.6

[a]Activities were assayed as described in Section 2. RMS deviations were calculated on the basis of two or three experiments.

react with amino moieties by mixing MTG and FTG, as compared with MTG alone. It should be noted that the MTG variants with higher activity and the mixing of more than two kinds of TGases work complementarily, since the reaction rate increased in the former case, while the number of glutamine residues that react with TGases increased in the latter case. In addition to the MTG variants with higher activity, combining more than two kinds of TGases with different substrate specificities is also expected to improve the physical and textural properties of many food products, as compared with the native TGase.

In summary, the mutants with higher activity, del1–2, del1–3 and S2R, in addition to Ser-MTG, were produced by site-directed mutagenesis on the basis of extracting flexible residues affecting the active site by NMR. This suggests that a strategy for identifying flexible regions in enzymes using NMR would be effective for protein engineering. It is possible that the highly flexible regions are not primarily important for the global fold. Therefore, these regions can be chosen as primary candidates for replacement and deletion, as long as they are close to the active sites of enzymes. The MTG mutants thus obtained are expected to improve the physical and textural properties of many food products more effectively, as compared with the wild-type MTG.

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